Applied adenine and the biosynthesis of cytokinins in maize stems

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No evidence for the synthesis of free cytokinins from adenine was obtained when apical and basal maize stem tissue was aseptically incubated in a medium containing [8-^{14}C] adenine. These findings are discussed in relation to the occurrence of high levels of endogenous cytokinin in intact maize shoots and the fact that adenine is considered to play a central role in cytokinin biosynthesis.
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Geen bevestiging vir die sintese van vrye sitokiniene vanaf adenien kon bepaal word toe apikale en basale stingelweefsel van mielies asepties in 'n [8-^{14}C] adenien-bevattende medium geïnkubeer is nie. Hierdie bevindings word bespreek in die lig daarvan dat hoë konsentrasies natuurlike sitokiniene in intakte mieliestingels voorkom en dat daar aanvaar word dat adenien 'n belangrike rol in sitokinien-biosintese speel.
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Introduction

Although cytokinin-like activity has been detected in maize roots on a number of occasions (Van Staden & Smith 1978; Van Staden & Forsyth 1984a, 1986) and this could indicate that these organs are sites of cytokinin biosynthesis, experiments with labelled adenine and adenosine, the presumptive precursors of these hormones, have not supported this hypothesis (Van Staden & Forsyth 1984a, 1986). This could mean that either the maize root system is not a major site of cytokinin production, or that the biosynthetic pathway does not involve adenine monomers in a similar way as it does in cytokinin autonomous shoot material (Chen *et al.* 1976; Stuchbury *et al.* 1979). Within the maize plant, the stems, and in particular the intercalary meristems, could serve as a source of cytokinins. This is supported by the finding that cytokinins are present in relatively high concentrations in maize stems (Hansen *et al.* 1984), the highest levels being detected in the apical internodes with lower levels in the basal internodes. Within the lower internodes a distinct asymmetry, with higher levels in the regions which included the nodes, was recorded. As it may well be an over-simplification to assume that roots necessarily produce the total cytokinin supply of plants, attempts were made to establish whether isolated maize stem tissue had the capacity for adenine incorporation into the free cytokinins. Such incorporation has been shown to occur in pea stems (Chen *et al.* 1985).

Materials and Methods

Plants of *Zea mays* cv. Silver King were grown in pots under natural conditions for 2 months. After removal of the leaves and the leaf sheaths, the stems of these plants were divided into apical and basal portions. The stem samples, which included both nodes and internodes, were sterilized with 3,5% NaOCl for 4 min and then rinsed three times in sterile distilled water. The sterilized material was transferred to a half-strength Miller's (1965) nutrient medium containing 1% sucrose and no hormones. [8-^{14}C] Adenine (specific activity 14,3 GBq m mol^{-1}, Amersham) was added to the medium. The cultures were maintained at a low light intensity at 25 ± 2°C on a rotary shaker for 5 days.

At the completion of the culturing period the contents of each culture vessel were homogenized with sufficient ethanol to give a final volume of 80% ethanol, and then allowed to extract at 5°C for 12 h. After filtration the extracts were reduced to dryness *in vacuo* at 35°C and then redissolved in 100 cm^3 80% ethanol. These ethanolic extracts were extracted for cytokinins using Dowex 50 cation exchange resin (Van Staden 1976). Both the Dowex 50 extracts and the washes

(aqueous phase) obtained were taken to dryness and subsequently redissolved in 5 cm^3 80% ethanol. A small aliquot (100 mm^3) of the respective aqueous and Dowex extracts was streaked onto Whatman No. 1 chromatography paper and separated in a descending manner with *iso*-propanol: 25% NH_4OH: water (10:1:1 v/v). After drying, the chromatograms were divided into 10 equal Rf fractions which were incorporated into scintillation vials. Methanol (1 cm^3) and scintillation cocktail (8 cm^3) (Hutton & Van Staden 1982) were added to each vial whereafter the radioactivity was determined with a Beckman LS 3800 scintillation counter. The remainder of the extracts were reduced to 2 cm^3 and then fractionated on a Sephadex LH-20 column (2,5 × 93 cm) using 10% methanol as eluant. Fractions of 40 cm^3 were collected and 1 cm^3 aliquots of each fraction were used for the detection of radioactivity by adding 4 cm^3 Beckman Ready-Solve EP. The remainder of each fraction was dried in a stream of air and stored for subsequent enzymatic or chemical treatment and TLC separation. For TLC, control and treated samples were spotted onto silica gel 60 F-254 plates (20 × 20 cm) together with adenine, adenosine, zeatin and ribosylzeatin as markers. The plates were subjected to two-dimensional ascending chromatography. *n*-Butanol: acetic acid: water (12:3:5 v/v) was used in the first direction and *n*-butanol: 25% NH_4OH: water (6:1:2 v/v upper phase) in the second direction. The UV-fluorescing spots co-eluting with the authentic markers were scraped off the plates, incorporated into scintillation vials and 1 cm^3 of methanol and 4 cm^3 POPOP (Hutton & Van Staden 1982) added to each. These samples were then counted for radioactivity. The rest of the plates were then divided into 25 equal blocks and the radioactivity in each block determined as described above.

Results and Discussion

The apical and basal stem material were incubated in similar amounts of radioactivity. Despite this the recovery of radioactivity from the basal material was considerably less than that from the apical tissue (Figure 1). As both the medium and the plant material were extracted it can only be concluded that a higher proportion of the radioactivity applied was incorporated into the non-soluble fraction of the basal stems. The relative ratios of total radioactivity recovered from the aqueous and Dowex 50 fractions however, did not vary much (Table 1). In both instances most radioactivity was present in the aqueous extracts. Two peaks of radioactivity were detected in all the extracts. One was polar and occurred at Rf 0 – 0,2 while the second co-chromatographed with adenine and adenosine which did not separate with the solvent system used. No radioactivity was detected in those parts of the chromatogram where zeatin, isopentenyladenine or their ribosides normally occur (Figure 1).

Fractionation of the aqueous extracts on Sephadex LH-20 yielded two polar radioactive peaks (Figure 2A and C). In both the apical and basal stems more than 95% of the radioactivity was associated with a peak which had an elution volume of 320 to 440 cm^3. The position of this peak did not shift following treatment with alkaline phosphatase (Van Staden *et al.* 1972) or after acid hydrolysis with HCl (Dyson *et al.* 1972). It would thus appear that the compound concerned is not a cytokinin nucleotide. The smaller more polar peak (240 – 320 cm^3) detected in the aqueous extracts was also unaffected by alkaline phosphatase or acid hydrolysis.

The Dowex 50 extracts from both the apical (Figure 2B) and basal (Figure 2D) stem tissue yielded five radioactive peaks following fractionation on Sephadex LH-20. These results

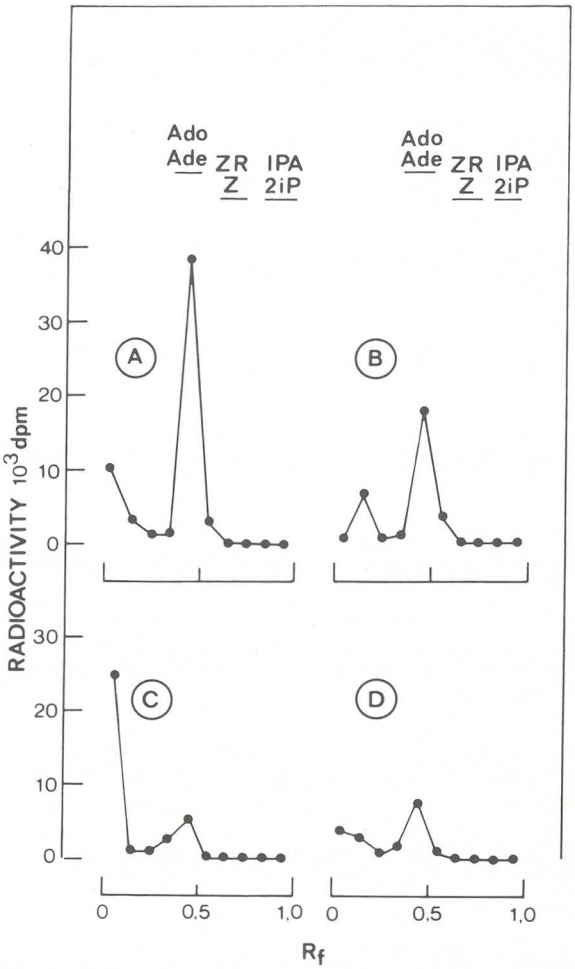

Figure 1 Radioactivity (dpm) detected in the aqueous (A & C) and Dowex (B & D) extracts of apical (A & B) and basal (C & D) maize stem tissue cultured in the presence of [8-^{14}C] adenine for 5 days. The extracts were separated with *iso*-propanol: 25% NH_4OH: water (10:1:1 v/v) on Whatman No. 1 paper, and the radioactivity in each Rf zone determined. Ado — adenosine; Ade — adenine; ZR — ribosylzeatin; Z — zeatin; IPA — *iso*-pentenyladenosine; 2iP — *iso*-pentenyladenine.

Table 1 Distribution of recovered radioactivity (expressed as a % of the total dpm detected) in the different fractions of apical and basal stems of maize following incubation with [8-^{14}C] adenine for 5 days

	Fraction	
Material	Aqueous	Dowex 50
Apical stems	62,4	37,4
Basal stems	65,0	35,0

were similar to those obtained when labelled adenosine was supplied to rooting leaf explants of bean (Van Staden & Forsyth 1985). None of the detected radioactive peaks co-eluted with any of the cytokinin markers used. No radioactive peaks were detected once 1000 cm^3 of eluant had passed through the column. Most radioactivity in the Dowex 50 extracts was associated with the two polar peaks which had elution volumes of 200 – 280 cm^3 and 320 – 440 cm^3. The chromatographic behaviour of these polar peaks was not affected by alkaline phosphatase treatment or acid hydrolysis. It would thus appear that the radioactive compounds do not represent cytokinin conjugates. The third peak (elution volume 440 – 560 cm^3) did not respond to either of the two treatments described above and was also not affected by treatment with

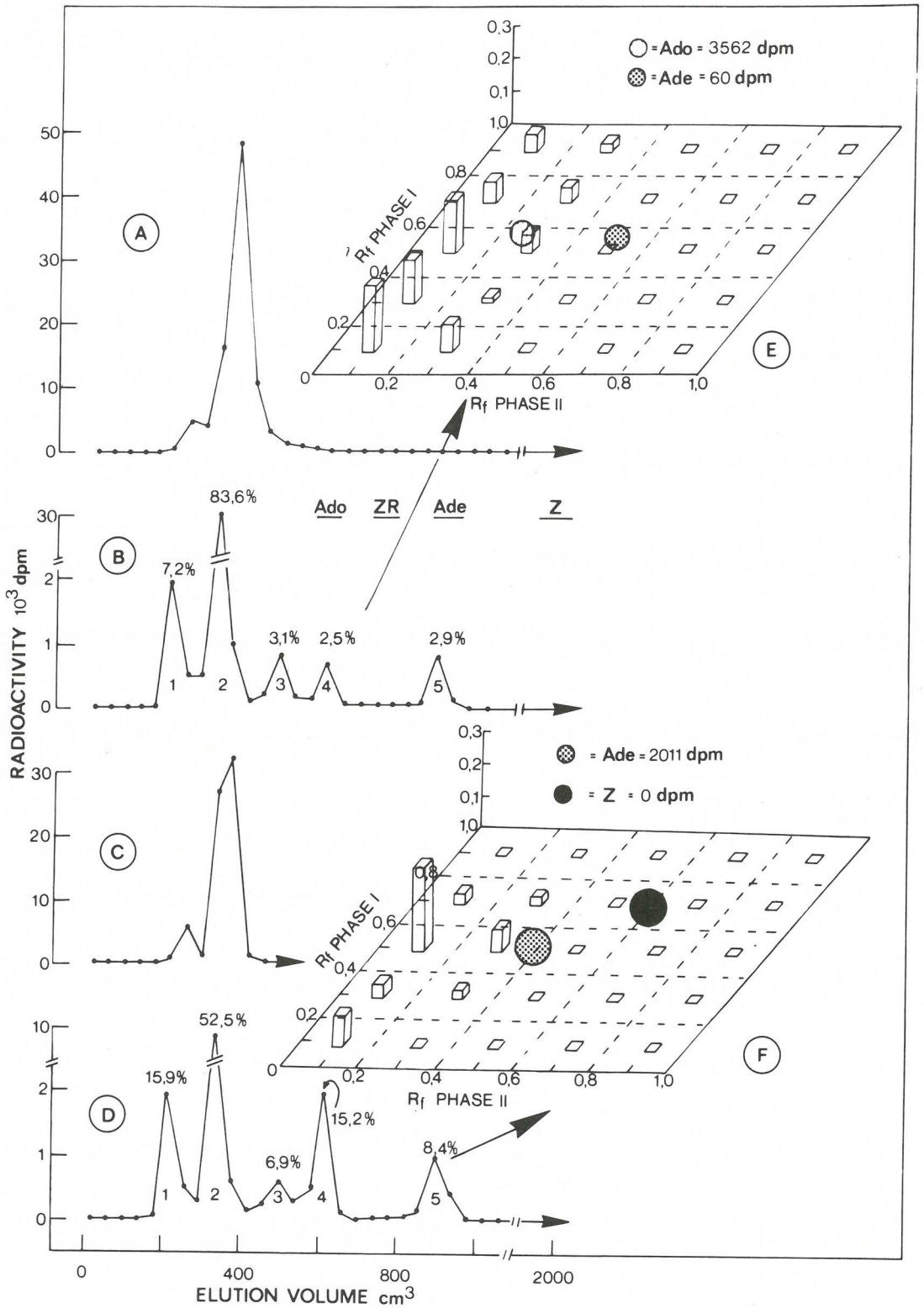

Figure 2 Radioactivity (dpm) detected in the aqueous (A & C) and Dowex (B & D) extracts of apical (A & B) and basal (C & D) maize stem tissue cultured in the presence of [8-^{14}C] adenine for 5 days. Extracts were fractionated on a Sephadex LH-20 column using 10% methanol as eluant. Of each 40 cm^3 fraction collected, 1 cm^3 was used for radioassay purposes. Aliquots from the peaks which co-eluted with adenosine (E) and adenine (F) were separated two-dimensionally on TLC plates and the radioactivity recorded. Ado — adenosine; Ade — adenine; ZR — ribosylzeatin; Z — zeatin; IPA — *iso*-pentenyladenosine; 2iP — *iso*-pentenyladenine.

β-glucosidase (Van Staden 1976). The peak which co-eluted with adenosine gave a single radioactive peak when separated by TLC (Figure 2E). After acid hydrolysis all the radioactivity shifted and co-chromatographed with adenine. This indicates that part of the applied adenine was converted to adenosine. The fifth peak co-eluted with adenine following separation on thin layer plates (Figure 2F).

Following earlier reports that labelled adenine was not incorporated into the free cytokinins of aseptically cultured maize roots (Van Staden & Forsyth 1984a, 1986) and the fact that the maize stem contained relatively high levels of these hormones (Hansen *et al.* 1984), it was suggested that meristematic regions of the stem may contribute to the free cytokinins in this plant (Van Staden & Forsyth 1986). This suggestion is consistent with the findings that cytokinins were produced *de novo* when adenine or adenosine was supplied to

autonomous tobacco callus (Chen *et al.* 1976; Burrows 1978; Nishinari & Syôno 1980) and *Vinca* crown-gall material (Stuchbury *et al.* 1979; Palni 1984). Biosynthesis in this case would necessitate the transfer of the isopentenyl group to adenine or adenosine and the subsequent hydroxylation of the products to form zeatin and ribosylzeatin which could be interconverted (Nishinari & Syôno 1980). In the present investigation and in others where 'normal' plant tissue such as developing lupin pods (Van Staden & Choveaux 1981), maize roots (Van Staden & Forsyth 1984a), rooting leaf cuttings of bean (Van Staden & Forsyth 1985) and intact bean plants (Maass & Klämbt 1981) were used, no support for *de novo* synthesis of cytokinins was found. The only positive results with 'normal' plant tissue were reported for tomato roots (Van Staden & Forsyth 1984b) and recently for pea roots and stems and carrot root tissue (Chen *et al.* 1985). Maass & Klämbt (1981) reported that cytokinin biosynthesis could occur via the turnover of poly- and oligonucleotides which would involve RNA-hydrolysis. The available evidence seems to indicate that cytokinins may well be produced by different routes which involve independent enzyme systems (Maass & Klämbt 1981). In order to elucidate the biosynthetic pathway(s) it is essential that more attention be given to the enzymatic reactions concerned.

Acknowledgements

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